Mitochondrially Targeted p53 Has Tumor Suppressor Activities In vivo

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Abstract

Complex proapoptotic functions are essential for the tumor suppressor activity of p53. We recently described a novel transcription-independent mechanism that involves a rapid proapoptotic action of p53 at the mitochondria and executes the shortest known circuitry of p53 death signaling. Here, we examine if this p53-dependent mitochondrial program could be exploited for tumor suppression in vivo. To test this, we engage Eμ-Myc transgenic mice, a well-established model of p53-dependent lymphomagenesis. We show that exclusive delivery of p53 to the outer mitochondrial membrane confers a significant growth disadvantage on Eμ-Myc–transformed B-cells of p53-deficient or alternate reading frame–deficient genotypes, resulting in efficient induction of apoptosis and impinged proliferation. Conversely, normal cells from thymus, spleen, and bone marrow showed poor infectivity with these viruses. This proof-of-principle experiment shows that exclusive reliance on the direct mitochondrial program exerts a significant tumor suppressor activity in vivo. Our in vivo data on the direct mitochondrial apoptotic p53 program lays the groundwork to further investigate its efficacy and safety and to address its possible therapeutic value in the future. (Cancer Res 2005; 65(21): 9971-81)

Introduction

The basis of the apoptotic and tumor suppressor p53 activities lies in its pleiotropic action, involving transcription-dependent and transcription-independent functions (1). Previous studies showed that p53 responds to a broad range of death stimuli by rapid stabilization and activation, and mediates cell death primarily via the mitochondrial pathway. Thus, p53 acts as a transcription factor of proapoptotic target genes, such as PUMA, Noxa, Bax, Bid, and p53AIP1, which all reside and/or act at the mitochondria (2–8).

Aside from its nuclear function, we previously described a direct apoptotic p53 program at the mitochondria. In response to various death stimuli, such as genotoxic drugs, γ-irradiation, and hypoxia, a fraction of induced wild-type p53 protein rapidly (within 30-60 minutes) translocates to mitochondria in primary, immortal, and transformed cells in culture (1, 9, 10). Moreover, induced p53 can physically interact with and form inhibitory complexes with antiapoptotic Bcl-XL and Bcl2 proteins via its p53 DNA-binding domain (1). Mitochondrial p53 induces Bak oligomerization, permeabilizes the outer mitochondrial membrane, and promotes the release of cytochrome c, leading to caspase-3 activation and cell death (1). Of note, this direct mitochondrial p53 program participates in the physiologic p53 response after DNA damage in vivo in normal mice (11). However, tumor-derived missense p53 mutants concomitantly lose or compromise their ability to interact with Bcl-XL and to promote cytochrome c release. Therefore, such mutants may represent “double hits,” eliminating the transcriptional as well as the direct mitochondrial function of p53 (1). Together, this suggests that the mitochondrial pathway can participate in tumor suppression in vivo.

Importantly, in p53-deficient cancer cell lines in vitro, mitochondrial targeting of p53 at least partly phenocopies nuclear p53 function in cell killing. Although mitochondrially targeted p53 bypasses the nucleus, it is sufficient to launch effective apoptosis and colony suppression directly from this platform (1, 9). Here, we test this novel pathway in vivo. We show as a proof-of-principle that p53 that has been deliberately excluded from the nucleus and targeted to the outer mitochondrial membrane efficiently kills primary lymphoma cells in mice and thus contributes to suppression of tumorigenesis in vivo.

Materials and Methods

Retroviral constructs. The replication-defective mouse stem cell virus MSCV-GFP-Bla was constructed by cloning green fluorescent protein (GFP) fused with basicidin S-deaminase (provided by M. Hayman, State University of New York, Stony Brook, NY) downstream of the polynucleotide in the MSCV backbone. The expression of GFP-Bla is driven by a separate cytomegalovirus (CMV) promoter. The following cDNAs were subcloned into this vector at EcoRI/NotI sites: human wild-type p53 (Nuclp53) or p53R175H (Nuclp53R175H; see Table 2); human wild-type p53 fused at its COOH terminus to the transmembrane domain of Bcl-XL (p53CTB) or Bcl2 (p53ICTM), respectively; or to the endoplasmic reticulum leader sequence of cytochrome b5 (p53ER). An additional control to rule out nonspecific effects of the transmembrane domain of Bcl-XL, enhanced GFP (EGFP) fused at its COOH terminus to the transmembrane domain of Bcl-XL (peptide sequence SRKGQERFNRWFLTGMTVAGVVLLGSLFSRK) was subcloned into the same vector (EGFP-TMBlcxl). Retroviral stocks were produced in PhoenixE cells.

Cells and tissue culture. p53−/− alternate reading frame (ARF)+/+ and p53+/− ARF−/− Eμ-Myc lymphomas were harvested from tumor-bearing lymph nodes of transgenic mice (C57BL/6-TgMyc). All animal studies were approved by the Stony Brook University Institutional Review Board. Independent isolates derived from six mice were used. Cell suspensions were plated on mitomycin-arrested NIH3T3 feeders and grown in 45% Iscove’s medium, 45% Dulbecco’s medium, and 10% fetal bovine serum (FBS) before spinoculation. Aliquots of cells were treated with Adriamycin (0.34 μmol/L) for the indicated times in some experiments.

Mouse embryonic fibroblasts (MEFs) were prepared from 14.5-day embryos (C57BL/6). p53−/− MEFs were derived from Trp53tm1Tyj mutant mice. ARF−/− MEFs were a gift from C. Sherr (Department of Biochemistry, St. Jude Children’s Research Hospital, Memphis, TN). MEFs and NIH3T3 were maintained in DMEM plus 10% FBS. In vitro growth of infected lymphoma cells was measured by plating equal numbers in triplicate on equal numbers of feeders, with daily counting of nonadherent
cells by Coulter counter and fluorescence-activated cell sorting (FACS). For \textit{in vitro} apoptosis studies, samples were initially adjusted to 65% of GFP-positive cells. For lymphoma cells, terminal deoxynucleotidyl transferase–mediated nick end labeling (TUNEL; Roche, Nutley, NJ) was done 36 hours after transduction without additional DNA damage. Apoptosis was quantified by calculating the fold increase over vector in the proportion of red cells that were also green (TUNEL/GFP) from three representative images, using the Automatic Measurement Program of AxioVision v4.3. This software tool analyzes the total area pixel intensities and densities of a color per image.

For MEFs, E1A-sensitized cells were retrovirally transduced, then selected in blasticidine for 4 days to obtain 100% GFP positivity and either left untreated or treated with adriamycin (0.34 \textmu mol/L) for the indicated times.

Cells from normal bone marrow, spleen, and thymus were harvested from C57BL/6 mice, put into culture on ST3 feeder layers, and spinoculated thrice with 8-hour interval, starting on the same day. GFP expression was quantitated by FACS on days 2, 4, 6, and 8 postinfection. Parallel cultures were immunophenotyped by FACS using the following cell markers: B220 (CD45R), IgM, MAC1 (CD11b), Thy1 (CD90), and erythroid marker TER-119 (all from BD PharMingen, San Diego, CA).

\textbf{Protein expression.} Whole cell lysates (30-70 \mu g) were immunoblotted with antibodies specific for p53 (mouse-specific CM-5, Vector, Burlingame, CA; human-specific DO-1 and mouse and human specific Pab 421, Calbiochem, San Diego, CA), p21 (SXM30, PharMingen), PUMA (AbCam, Cambridge, MA); p19ARF (AB80-50, AbCam); p21 (F5), MDM2 (SMP14), BclXL/xs (S18), E1A (135-5), cleaved caspase-3 (Asp175, does not cross-react with procaspase-3; Cell Signaling Technology) and proliferating cell nuclear antigen (PCNA; all from Santa Cruz Biotechnology, Santa Cruz, CA).

\textbf{Immunofluorescence.} Lymphoma cells were cytospon onto slides; ST3 cells were grown in eight-well chamber slides. Cells were fixed in 4\% paraformaldehyde for 20 minutes and permeabilized with PBS/0.5\% Tween 20 for 5 minutes. Slides were blocked in 10\% normal goat serum, followed by 2 hours incubation with CM-1 (1:500; human specific p53; Vector) and either cytochrome c (1:200; clone 2G8, gift of R. Jemmerson, Department of Neurosciences, University of Minnesota, Minneapolis, MN) or mt hsp70 (1:25; ABR Affinity Bioreagents, Golden, CO) to detect mitochondria. For p53ER, p53 was detected with DO-1 and endoplasmic reticulum was detected with anticalretilcin (ABR Affinity Bioreagents). Anti-mouse IgG and anti-rabbit IgG were used for controls. Slides were incubated in Cy5- and tetramethylrhodamine isothiocyanate–conjugated secondary antibodies for 1 hour. Cells were viewed in a confocal laser microscope (Zeiss LSM 510). Tumor sections were stained with DO-1 by immunoperoxidase.

\textbf{Mitochondrial membrane potential.} To measure mitochondrial membrane potential as an indicator of mitochondrial dysfunction, freshly transduced whole cells were incubated in 300 nmol/L Mitotracker Red CMXRos (Molecular Probes, M-7512) added to the culture for 30 minutes at 37\(^\circ\)C, washed, and analyzed by FACScan. For each curve, 10,000 GFP-positive events were acquired.

\textbf{Figure 1.} Characterization of lymphoma isolates used in \textit{in vitro} and \textit{in vivo} studies. A, general scheme of murine stem cell virus MSCV-p53 CMV-GFP-blasticidin. p53 inserts were either nuclear human wild-type p53 (Nucp53) or the tumor-derived R175H mutant (Nucp53 R175H), or human wild-type p53 fused at the COOH terminus to the transmembrane domain of human BclXL (p53CTB) or Bcl2 (p53CTM), respectively. The control virus lacked the p53 insert. For another control, EGFP was fused at its COOH terminus to the isolated transmembrane domain of BclXL (EGFP-TM-BclXL). B and C, similar expression levels among the various p53 constructs. Retrovirally driven expression of nuclear and mitochondrially targeted p53 in NIH3T3 cells (B) and in p53 null and ARF null primary lymphoma cells (D). Immunoblot analysis for p53. PCNA was used as loading control. D, transduced levels of targeted p53 do not exceed the physiologic range. Endogenous p53 protein levels of ARF null lymphomas after adriamycin (0.34 \textmu mol/L for 4 hours) are higher than transduced levels of p53CTM and p53CTB in p53 null lymphomas. Immunoblot for p53 using the mouse and human p53-specific monoclonal antibody PAb 421. The fusion proteins migrate slightly slower. PCNA was used as loading control. E, viral transduction efficiencies of cells shown in (D), as determined by coexpressed GFP marker via FACS analysis 48 hours after transduction. A representative set with p53 null lymphoma cells is shown. Similar efficiencies were obtained with ARF null cells. Uninfected parental cells were used to calibrate the FACS instrument for the M2 region. After adjustment to a 40:60 ratio with parental cells, these cells were then immediately injected into nontransgenic recipient mice.
In vivo competition experiments. Three protocols using GFP-positive ratios of 40:60, 75:25, and 90:10 were used as described in Results. FACSSorted cells were cultured for 3 days before injection to verify sterility. Lymphoma cells (1 x 10^6) in 100 μL PBS were injected into the tail vein of syngeneic, nontransgenic recipient mice. After 26 to 30 days, animals had developed palpable lymphomas in the cervical and/or inguinal, axillary, and retroperitoneal regions. Occasionally, extranodal lymphomas were seen. Tumors from each recipient were pooled and analyzed by flow cytometry to determine residual GFP expression. To verify histopathology, each tumor was processed for H&E. In no case were inflammatory lymphocytic infiltrates within or around the tumors observed. Likewise, damage of normal tissue components, including vasculature and stroma, was not present. For apoptosis assays in vivo, lymphomas were fixed and stained by TUNEL with Hoechst counterstaining. Alternatively, 2 x 10^6 transduced lymphoma cells were injected s.c. into normal syngeneic mice and animals sacrificed 2, 4, 8, and 12 days later. Injection sites were harvested and processed for TUNEL/H&E and p53 immunoperoxidase staining with DO-1.

Results

p53CTB and p53CTM target to mitochondria. We and others recently reported that a fraction of induced p53 translocates to the mitochondria of apoptosing tumor cells (9, 12). Moreover, targeting

Figure 2. p53CTB targets to mitochondria. Immunofluorescence analysis. p53CTB (stained in red with human p53 specific antibody CM-1) colocalizes with cytochrome c (stained in blue with anticytochrome c antibody 2G8). No nuclear staining is detectable. Transduced cells are marked by GFP. ST3 cells are shown. Identical results were obtained in lymphoma cells and H1299 cells. Retrovirally expressed Nuclp53 shows a nuclear distribution.

Figure 3. Mitochondrially targeted p53 proteins lack transcriptional activity. A, p53−/− MEFs were transduced with empty vector, p53CTM, or p53CTB and either left untreated or treated with 0.34 μmol/L adriamycin for the indicated times. Uninfected wild-type MEFs are the positive control. Immunoblot analysis for p53, p21Waf1, and PUMA. B, p53−/− lymphoma cells (isolate 5) were transduced with empty vector, p53CTM, or p53CTB to 65% GFP positivity and left untreated or treated with 0.34 μmol/L adriamycin for the indicated times. Wild-type p53 harboring ARF null lymphoma cells were used as positive control. Immunoblot analysis for p53, p21Waf1, PUMA, Bax, and MDM2 (not shown). PCNA was used as loading control in (A) and (B).
p53 to mitochondria using mitochondrial import leader fusions is sufficient to launch apoptosis in p53-deficient cells (1). To determine if mitochondrially targeted p53 retains its proapoptotic and tumor-suppressive properties in vivo, we generated a series of murine stem cell viruses expressing GFP and either conventional nuclear p53 (Nuclp53) or two versions of mitochondrially targeted p53 (p53CTM and p53CTB). Control virus lacked p53 inserts (Fig. 1). p53CTM is a COOH-terminal fusion of human wild-type p53 with the transmembrane domain of Bcl2. We previously showed that p53CTM fails to localize to the nucleus but instead targets primarily to mitochondria and promotes apoptosis and colony suppression in p53 null human cancer cells (1). p53CTB is a COOH-terminal fusion of human wild-type p53 with the transmembrane domain of Bcl-xL. Native Bcl-xL is a resident

Figure 4. Mitochondrial p53 promotes apoptosis in vitro. A, wild-type MEFs, p53−/− MEFs, and ARF−/− MEFs (all 129xBL6 genotype; passages 3-5) were sensitized by transduction with E1A-encoding retroviruses. Subsequently, cells were transduced with empty vector, p53CTM, or p53CTB and enriched to 100% purity for 3 days in blasticidin. Cells were then treated with 0.34 μmol/L adriamycin for 6 or 12 hours or left untreated, and TUNEL assays were done. B, primary p53 null lymphoma cells, 72 hours after infection with the indicated viruses, were further enriched to >90% GFP positivity by FACS sorting, immediately followed by TUNEL assay. Alternatively, cells were enriched to >90% GFP positivity by blasticidin selection for 2 more days before TUNEL assay. Representative images of one of three independent experiments are shown. The average fold increase compared with vector is indicated on the left.

C, example of raw FACS data used to generate the graph in (D), indicating a drop in GFP intensity over time in p53 null lymphoma cells infected with p53CTM virus.
mitochondrial protein and specifically localizes to the mitochondrial outer membrane in unstressed and stressed cells (13, 14). Mitochondrial localization of Bcl-xL is exclusively mediated by the COOH-terminal transmembrane domain (14).

In primary p53-deficient or ARF-deficient lymphoma isolates (examples in Supplementary Fig. S1; subsequently also used in vivo) as well as in NIH3T3 cells, protein levels of transduced Nuclp53 were similar to levels of transduced p53CTM and p53CTB (Fig. 1B and C). Moreover, the GFP intensity profiles in transduced p53-deficient or ARF-deficient lymphoma cells were very similar between empty vector, Nuclp53, p53CTM, and p53CTB (M2 region in Fig. 1E; data not shown). This indicates that targeted p53 does not affect the level of GFP expression in a manner different from nuclear p53, thus validating GFP as a readout for cell survival. Of note, transduced levels of targeted p53 were within physiologic range because protein levels of p53CTM and p53CTB in p53 null lymphomas were actually lower than endogenous p53 levels in ARF null lymphomas stressed with adriamycin (Fig. 1D). Moreover, p53CTB and p53CTM levels are comparable with the stress-induced endogenous p53 levels of normal irradiated thymocytes (Supplementary Fig. S2). Flow cytometric examination of cells showed that infection efficiency was around 40% for all constructs, as indicated by GFP positivity (Fig. 1E). Localization studies showed that in contrast to the nuclear localization of transduced wild-type p53 (Supplementary Fig. S3), p53CTB localized exclusively to mitochondria in several cell types (MEF, NIH3T3 cells, and human H1299 carcinoma cells; Fig. 2). p53CTM exhibited a predominant mitochondrial localization (Supplementary Fig. S4A) plus some minor localization to endoplasmic reticulum (Supplementary Fig. S4B) consistent with native Bcl2 localization (14). To rule out that the small endoplasmic reticulum–localized subfraction of p53CTM contributes to apoptosis, we deliberately targeted p53 to the endoplasmic reticulum by replacing the Bcl2 domain with the endoplasmic reticulum leader sequence of cytochrome b5.

As expected, in contrast to p53CTB and p53CTM, p53ER lacked any apoptotic ability in p53 null H1299 and SaOS-2 cells, indicating that the apoptotic ability of p53CTM is solely due to its mitochondrial action (Supplementary Fig. S4C).

**Mitochondrially targeted p53 lacks transcriptional activity.** p53CTB and p53CTM proteins were undetectable in nuclei of all cells tested, including MEFs, NIH3T3, and H1299 cells (Fig. 2; Supplementary Fig. S4A and B and data not shown). To definitively rule out residual transcriptional activity of these proteins, we analyzed their ability to induce endogenous target genes of the apoptotic and arrest category. In contrast to stressed endogenous p53 in wild-type MEFs or retrovirally transduced wild-type p53, p53CTM and p53CTB were unable to induce p21Waf1, PUMA, or Bid in p53 null MEFs irrespective of the absence or presence of Adriamycin (Fig. 3A; data not shown). More importantly, p53CTM and p53CTB also lacked transcriptional activity in p53 null lymphoma cells, as indicated by lack of induction of p21Waf1, PUMA, Bax, and MDM2 even after further challenge by adriamycin. In contrast, ARF null lymphoma cells, which harbor endogenous wild-type p53 that is fully functional for a DNA damage response (as opposed to oncogenic challenge), induce these target genes (Fig. 3B; data not shown). Parenthetically, p53CTM/CTB—but not vector-transduced lymphomas—also showed increased levels of Bcl-xS isoforms, proapoptotic alternate splice products of the Bcl-x gene, a marker for activation of the mitochondrial death pathway (Supplementary Fig. S5A; refs. 15, 16). Thus, p53CTM/CTB expression in lymphoma cells induces an altered splicing ratio between Bcl-xL and Bcl-xS in favor of xS, and this contributes to mitochondrial dysfunction. We had also shown previously that p53CTM formed specific complexes with Bcl-xL and promoted apoptosis and colony suppression of Saos2 cells (17). To further show the negative effect of p53CTB and p53CTM on mitochondrial

| Table 1. In vivo cell death of p53 null B cells expressing mitochodrially targeted p53 |
|----------------|----------------|-------------|----------|-----------------|----------------|
| Retroviruses | Ratio of injected B-lymphoma cells, transduced/parental | Isolate ID injected (no. independent experiments) | No. mice with tumors | Average tumor weight (g) | Percentage tumor cells GFP-positive* at 28-30 d postinjection (%) |
| No preselection, no sorting | 40:60 | 2, 3, 4, and 5 (9) | 18 | 1.0 ± 0.2 | 30.8 ± 18 |
| Vector-GFP | 40:60 | 12 | 0.7 ± 0.3 | 0 |
| Nuclp53-GFP | 40:60 | 13 | 1.0 ± 0.4 | 0.4 ± 1.0 |
| p53CTM-GFP | 40:60 | 4 | 0.4 ± 0.1 | 0 |
| p53CTB-GFP | 40:60 | 3 | 0.7 ± 0.2 | 44.3 ± 3 |
| Blasticidin preselection | 75:25 | 3 | 0.5 ± 0.2 | 12 |
| Vector-GFP | 75:25 | 11 | 0.6 ± 0.4 | 1.1 ± 0.1 |
| Nuclp53-GFP | 75:25 | 14 | 1.1 ± 0.3 | 70 ± 10 |
| CTMp53-GFP | 90:10 | 7 | 1.3 ± 0.3 | 14 ± 14 |

* ± SD.
1P < 0.0005 compared with vector group.
1P < 0.0001 compared with vector group.

If the 13 animal series is expanded by one additional aberrant tumor, the percentage GFP-positive tumor cells at 28 days is 2.8 ± 2.4%. This tumor exhibited 34% GFP positivity, was genotyped as wild-type p53 by sequence, but expressed an abnormal p53 protein. Western blot analysis exhibited strong ubiquitin-like bands (higher molecular weight ladder) recognized by p53 antibody (see Supplementary Fig. S8A).

| p53 expression was lost in this tumor as judged by Western blot analysis (see Supplementary Fig. S8A). |
A
p53--/ lymphoma cells, 40% transduced cells initially injected i.v.

vector

uninf. control

2.2%

p53CTM

uninf. control

0.57%

p53CTB

uninf. control

1.0%

Nucl p53

uninf. control

0.05%

Mouse 1

uninf. control

0.05%

Mouse 1

uninf. control

0.09%

Mouse 2

uninf. control

0.56%

Mouse 2

uninf. control

0.26%

H&E

H&E

H&E

H&E

B
12 day lymphomas

H&E

Hoechst

TUNEL

p53

vector

p53CTM

p53CTB

p53--/ lymphoma cells, 85% transduced cells initially injected s.c.
In vivo \textit{Tumor Suppressor Activities of p53}

Figure 5. A, mitochondrially targeted p53 kills primary lymphoma cells \textit{in vivo}. Representative examples of FACS analyses of residual GFP expression in reconstituted lymphomas at day 28. Tumors were generated by tail vein injections of primary p53 null lymphoma cells (1 × 10^6) containing 40% virally transduced cells into nontransgenic recipients. Two representative animals each are shown. The uninfected parental cells served to calibrate the FACS instrument. Corresponding H&E sections confirm the histopathology of large cell lymphoma with severe nuclear atypia and apoptotic bodies. B and C, kinetics of mitochondrial p53-induced apoptosis \textit{in vivo}. \textit{B}, 12-day lymphomas generated by injection of transduced p53 null cells (1 × 10^6) into the dorsal skin of nontransgenic mice. A mixture of 85% transduced (after FACS sorting) and 15% parental cells were injected. \textit{In situ} tumor growth is shown by H&E staining, apoptotic activity is monitored by TUNEL staining, and residual p53CTM- and p53CTB-expressing lymphoma cells are identified by p53 immunoperoxidase staining with DO-1. Brisk apoptotic activity was seen in p53CTM and p53CTB tumors. C, example of a reconstituted lymphoma in a cervical lymph node harvested 28 days after tail vein injection of 90% transduced and 10% parental p53 null lymphoma cells. At that late time, terminal p53CTM and p53CTB tumors are “burnt out.” They have largely depleted their p53-expressing cell populations and, thus, are predominantly composed of uninfected competitor cells. Hence, FACS determination showed that this terminal p53CTM tumor contained only 2% residual GFP-positive cells.

function, we measured the mitochondrial membrane potential of freshly transduced p53 null lymphoma cells, using the well-established FACS analysis of cells stained with the potentiometric dye Mitotracker Red CMXRos (Supplementary Fig. S5B). We observed that the p53CTB and p53CTM curves were shifted to the right in a highly reproducible manner compared with vector alone, indicating hyperpolarization of the mitochondrial membranes of p53CTB- and p53CTM-transduced cells. Hyperpolarization of the mitochondrial membrane is one of the early markers of mitochondrial dysfunction (17). Together, these data strongly suggest that any apoptotic activity of p53CTB/CTM that we might see in subsequent experiments \textit{in vivo} is due to their direct action at the mitochondria and not due to a cryptic transcription-dependent p53 function.

Mitochondrially targeted p53 promotes apoptosis in primary lymphoma \textit{in vitro}. To show apoptotic activity of targeted p53, we first analyzed the killing ability of p53CTB and p53CTM \textit{in vitro} on primary MEFs and lymphoma isolates. Wild-type, p53 null, and ARF null MEFs were presented with cell death by retroviral transduction with E1A. After treatment with Adriamycin for 6 or 12 hours, mitochondrial p53CTB and p53CTM proteins increased apoptosis by -2- to 3-fold in all three genotypes, compared with vector alone (Fig. 4A; Supplementary Fig. S6).

Likewise, in p53 null lymphoma cells, short-term expression of p53CTM and p53CTB alone, in the absence of any DNA damage, increased apoptosis by 5.2- and 5.4-fold on average, respectively, over empty virus, as indicated by TUNEL assays, albeit this effect was somewhat weaker than that seen with nuclear wild-type p53 (5.8-fold; Fig. 4B and Supplementary Table S1). These levels of apoptosis seen by TUNEL (compared with empty vector) were also supported by mild differences in the levels of cleaved caspase-3, as shown by immunoblot analysis (Supplementary Fig. S5C).

When assayed over a period of 25 days in growth competition assays, the numbers of p53CTM- and Nuclp53-infected lymphoma cells both sharply declined. In contrast, control cells transduced with empty virus or EGFP-TM Bcl-xL (also called GFPCTM) dropped only slightly (Fig. 4C and D). On the other hand, cell cycle analysis by FACS showed no evidence for an antiproliferative effect of p53CTM expression in these cells (data not shown). Together, this indicates that mitochondrially targeted p53 leads specifically to lymphoma cell apoptosis rather than to a general cytotoxic effect.

Mitochondrially targeted p53 mediates tumor suppression in primary lymphomas \textit{in vivo}. E\textit{μ}-Myc transgenic mice overexpress the c\textit{-myc} oncogene in their B-cell lineage and develop pre-B and B-cell lymphomas within 4 to 6 months of age (18). This well-established transplanted tumor model has many advantages. Most importantly, test genes can be efficiently introduced \textit{ex vivo} by retroviral gene transfer into isolated primary lymphoma cells and injected into the tail vein of normal syngeneic recipient mice for lymphoma reconstitution. Tumor formation and aggressiveness depends on a disabled p53 pathway (19–24). Tumor apoptosis occurs via the mitochondrial pathway because Bcl2, which is not overexpressed in these lymphomas, produces multidrug resistance when forcibly overexpressed (20). In murine fibroblasts and lymphoid cells, loss of p53 is equivalent to loss of INK4a/ARF, the positive upstream regulator of p53, and both events greatly accelerate c\textit{-myc} lymphomagenesis (19). Compared with parental tumors, p53- null and ARF null lymphomas are highly invasive with severe apoptotic defects and marked resistance to chemotherapy (19). We isolated several independent primary lymphomas (four tumors of genotype p53\textsuperscript{−/−}/ARF\textsuperscript{+/+} and two tumors of genotype ARF\textsuperscript{−/−}/p53\textsuperscript{+/−}) from p53\textsuperscript{−/−} and ARF\textsuperscript{+/−} E\textit{μ}-Myc mice. After 2 days in culture, isolates were transduced with MSCV-GFP control virus or viruses expressing nuclear or mitochondrially targeted p53 (Supplementary Fig. S7). After 36 to 48 hours, these cells were then immediately injected into the tail veins of syngeneic normal recipient mice to assess the effect of mitochondrially targeted p53 on tumor burden \textit{in vivo}. In the context of p53 null lymphomas, this model provides a quantitative measure whether mitochondrial p53, as the sole source of cellular p53, has efficient tumor killing
actions in vivo to cause tumor suppression at natural sites. Thus, the rapidly reconstituted lymphomas in the recipient mice differed only by the presence or absence of p53 proteins (20). Importantly, mouse and human p53 are functionally completely interchangeable in vivo, because knock-in mice harboring the human p53 DNA-binding domain within a mouse gene backbone develop normally, show wild-type p53 responses to DNA-damaging agents and have wild type–like tumor suppression (25). Moreover, as shown in Fig. 2, human p53 fusion proteins expressed in mouse cells target properly to mitochondria.

All experiments were done as in vivo competitions between transduced and parental cells using three different protocols. In the first protocol, 40% of freshly transduced p53 null lymphoma cells, confirmed for GFP positivity at 36 hours (see Fig. 1E), were mixed with 60% GFP-negative parental lymphoma cells and immediately injected into recipients (1 × 10⁶ per mouse). Four weeks later, all reconstituted tumors from a given recipient were pooled and their residual GFP positivity determined by FACS (Table 1; examples in Fig. 5A). Eighteen mice injected with 40% empty virus-GFP yielded 30.8 ± 18% residual GFP-positive tumors, reflecting the ability of vector-transduced cells to survive in the bloodstream and proportionally contribute to lymphoma reconstitution. In sharp contrast, each of 12 control mice injected with 40% Nuclp53-expressing cells yielded 0% residual GFP positivity. Of note, the 13 mice receiving 40% p53CTM-GFP–expressing cells yielded tumors with only 0.4 ± 1.0% residual GFP-positive cells. The individual tumor GFP-positive scores of the p53CTM group were 0%, 0%, 0%, 0%, 0%, 0%, 0%, 0%, 0%, 0%, 0.6%, 1.5%, 3.5%, respectively (P < 0.0005 compared with empty virus group). An additional single mouse yielded tumors with 34% residual GFP positivity but analysis indicated that it expressed a functionally inactive p53 (see Supplementary Fig. S8A). Likewise, four mice injected with 40% p53CTB-GFP–expressing cells yielded tumors without detectable residual green cells (individual GFP scores were 0%, 0%, 0%, and 0%; P < 0.0005). Thus, all 13 mice yielded tumors with 0% GFP positivity and in aggregate, these data clearly confirm that mitochondrially targeted p53, as the sole source of cellular p53 in a mouse background, we used the tumor-derived inactive human mutant p53R175H. As expected, mice injected with a 75:25 ratio of Nuclp53(R175H)-transduced lymphoma cells yielded tumors with 51 ± 18% GFP positivity (n = 6; P < 0.0005 compared with empty virus), confirming that the suppression seen with targeted p53-expressing vectors is specifically mediated by p53CTB and p53CTM (Table 2).

Mitochondrially targeted and nuclear p53 cooperate in tumor suppression. Despite the presence of a wild-type p53 gene, ARF null lymphomas exhibit a severely impaired p53 activity toward oncogenic deregulation (19, 24). To test whether targeted p53 also kills these cells in vivo, two independent ARF null tumor isolates were used in the competitive 40:60 protocol (Supplementary Fig. S1; Table 3). Generally, we found a higher sensitivity to spontaneous and p53-induced cell death in ARF null lymphoma cells compared with p53 null lymphoma cells. Thus, mice injected with 40% empty virus-infected cell mixtures produced reconstituted tumors with 18 ± 13% GFP positivity (n = 14). In contrast, mice injected with 40% p53CTM-expressing cell mixtures produced tumors with only 0.2 ± 0.4% residual GFP positivity (n = 12; P = 0.0001). Moreover, mice injected with 40%
p53CTB-expressing cell mixtures produced tumors with only 0.4 ± 0.7% residual GFP positivity (n = 11; P = 0.0001). This result is identical to that from mice injected with Nuclp53-expressing cells (n = 13; 0.6% ± 0.6%). Thus, as in p53 null lymphomas, ARF null lymphomas expressing mitochondrially targeted p53 show a dramatic in vivo suppression. Moreover, in these cells, the mitochondrial p53 program can functionally complement the endogenous p53 program.

Mitochondrially targeted p53 mediates tumor suppression by inducing apoptotic cell death. To obtain in vivo proof of p53 CTB/CTM-induced apoptosis in lymphoma tissues, tumors were assayed by TUNEL staining. We chose an early time point during lymphoma reconstitution, because once tumors have reached a certain size, secondary factors such as necrosis due to insufficient blood supply render interpretation unreliable. To this end, transduced lymphoma cells, sorted to 85% GFP positivity and mixed with 15% parental cells, were s.c. injected into recipient mice to allow easy observation of tumor growth (18). Twelve days later, s.c. sites were harvested and in situ tumor growth was confirmed by H&E staining. As expected, a much higher degree of apoptosis was present in lymphomas generated by p53CTM- and p53CTB-expressing cells, indicated by large numbers of TUNEL-positive cells and apoptotic bodies compared with lymphomas derived from empty virus cells (Fig. 5B). Conversely, at that time point, the p53CTM- and p53CTB-derived lymphomas still contained a viable, albeit drastically decimated (from the initial 85%) subpopulation of mitochondrial p53-expressing tumor cells (Fig. 5B). On the other hand, at 28 days postinjection, the composition of reconstituted lymphomas represented the “winning” cell type at end point, because all mice die from their malignancy within 1 to 3 days if not sacrificed. Thus, at that late time, terminal p53CTM and p53CTB tumors have largely depleted their p53-expressing cell populations and instead were predominantly composed of uninfected competitor cells. Hence, tumors derived from p53CTM/CTB-injected mice have similar low background apoptotic activities as vector tumors because they are “burnt out” (Fig. 5C). Thus, the bulk of mitochondrial p53-driven tumor cell death occurs early in the course of lymphoma reconstitution, likely starting already in the injected circulating cell population before homing into lymph nodes.

**Lack of infectivity by p53CTB retrovirus in normal cells.** Whereas we successfully used retroviral gene transfer in this animal model, one critical issue with any kind of therapeutic approach aiming at introducing toxic proteins into cancer cells is how to specifically target cancer cells but not normal cells in vivo. Intratumoral injection of recombinant viruses has been the preferred route to maximize transduction of cancer cells and minimize infecting normal cells. To further begin to address this issue directly, we attempted to infect normal cells from young C57BL/6 mice including primary bone marrow cells, splenocytes and thymocytes with the same MSCV-based retroviruses used throughout this study (see Fig. 1A). However, overall, attempts to infect normal thymocytes, splenocytes, and primary bone marrow cells with the same retroviruses used to study the lymphomas were largely unsuccessful. Thus, normal cells and lymphomas behave differently in terms of retroviral transduction. As shown in Supplementary Table S2, wild-type splenocytes and thymocytes were not infectable in two and three independent experiments, respectively. Moreover, >80% of wild-type bone marrow cells were not infectable as indicated by only a small percentage of GFP-expressing cells at peak viral expression on day 4. At day 4, empty vector infected 14% B-lymphocyte–enriched (R2) and 10% macrophage-enriched (R3) fractions, whereas p53CTB infected 8% B-lymphocyte– and 2% macrophage-enriched fractions. Whereas the p53CTB-infected cells in the R3 (macrophage population) clearly persisted, they did not expand, as they did for the vector

### Table 2. Lack of in vivo killing of lymphoma cells expressing human mutant p53

<table>
<thead>
<tr>
<th>Retroviruses</th>
<th>Ratio of injected B-lymphoma cells, transduced/parental</th>
<th>Isolate ID injected (no. independent experiments)</th>
<th>No. mice with tumors</th>
<th>Average tumor weight (g)</th>
<th>Percentage tumor cells GFP-positive at 28-30 d postinjection (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclp53 (R175H)</td>
<td>75:25</td>
<td>6</td>
<td>0.3 ± 0.2</td>
<td>51 ± 18</td>
<td></td>
</tr>
</tbody>
</table>

**Table 3. In vivo cell death of ARF null B cells expressing mitochondrially targeted p53**

<table>
<thead>
<tr>
<th>Retroviruses</th>
<th>Ratio of injected B-lymphoma cells, transduced/parental</th>
<th>Isolate ID injected (no. independent experiments)</th>
<th>No. mice with tumors</th>
<th>Average tumor weight (g)</th>
<th>Percentage tumor cells GFP-positive* at 28-30 d postinjection (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vector-GFP</td>
<td>40:60</td>
<td>1 and 6 (2)</td>
<td>14</td>
<td>0.9 ± 0.4</td>
<td>18 ± 13</td>
</tr>
<tr>
<td>Nuclp53-GFP</td>
<td>40:60</td>
<td>13</td>
<td>0.33 ± 0.2</td>
<td>0.6 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>CTMp53-GFP</td>
<td>40:60</td>
<td>12</td>
<td>0.23 ± 0.1</td>
<td>0.2 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>CTBp53-GFP</td>
<td>40:60</td>
<td>11</td>
<td>0.37 ± 0.2</td>
<td>0.4 ± 0.7</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: Isolates 1 and 2 were used.

*SD.

†P = 0.0001 compared with vector group.
control R3 and for the vector and p53CTB infected R2-populations. In light of the inconclusive data on the R3-infected cells, we cannot firmly conclude that p53CTB fails to induce death in a small subfraction of normal bone marrow cells. A further indication for low infectivity of normal cells is the fact that we were unable to infect p53 null bone marrow cells with either empty virus or p53CTB. Taken together, the MSCV-based p53CTB virus seems to have absent or low infectivity of normal cells and the small percentage of normal cells that do get infected do not undergo apoptosis. These data is consistent with findings by Bossi et al. (26) that infection of primary bone marrow cells with a retrovirus encoding conventional wild-type p53 does not impair the ability of hematopoietic stem cells to reconstitute the bone marrow compartment of syngeneic, irradiated mice. Furthermore, simultaneous infection of leukemia and bone marrow cells with this retroviral wild-type p53 depleted the neoplastic but not the normal transplanted animals. These results show that exogenous wild-type p53 is controlled as tightly as the endogenous one in normal cells (26).

Discussion

Abrogation of p53-mediated apoptosis is a fundamental defect in human tumors and promotes chemoresistance; hence, its functional restoration is a premier therapeutic target in a "universal" cancer strategy. As a proof-of-principle, we show here that exploiting the shortest known circuitry of p53 death signaling (i.e., the direct transcription-independent mitochondrial p53 death program may have therapeutic potential in the future).

Up to now, efforts focused on restoring transcription-mediated p53 apoptosis. One venue tries to identify small molecules that structurally rescue tumor-derived p53 mutant proteins. Two prototype compounds, CP-31398 and PRIMA-1, can restore transcriptional p53 function in cell-based assays and slow tumor growth in nude mice. However, their mechanism of action is unclear (27, 28) and their in vivo efficacy is untested. Another venue focuses on supplying conventional wild-type p53 via gene therapy (29). Both the wild-type p53 and small molecule approach rely on the preserved ability of tumor cells to respond with transcriptional activation of p53 target genes. However, many human cancers have lost this prerequisite due to global epigenetic deregulation of their genome, leading to broadly aberrant gene silencing patterns (30). Moreover, recent genetic studies showed that at least some p53 mutants with reduced transcriptional activity retain substantial proapoptotic activity in response to various stresses (e.g., p53S15A, p53S23A, p53S389A, p53L25QW268; refs. 31–35). These data support the idea that p53 is able to induce apoptosis in a transcription-independent manner. As a transcription factor, p53 works as a homotrimer mediated via its COOH-terminal domain, which makes the protein vulnerable to dominant negative inhibition by endogenous p53 mutants that are expressed at high levels in cancer tissues. An additional vulnerability for dominant negative interference of ectopic p53 emanates from ΔN isoforms of p63 and p73, which are also frequently overexpressed in human cancers (36, 37).

p53-dependent apoptosis mainly uses the intrinsic mitochondrial pathway (38). Importantly, apoptosis relies upon a preassembled death machinery of protein and DNA-degrading enzymes that do not require transcription of new genes. Indeed, apoptosis can be triggered and proceed to its biochemical end point in nuclei-free cytoplasts (39–41). Our study shows that transcriptional p53 restoration might be dispensable for tumor killing in vivo. Instead, tumor cell suppression can be achieved by exploiting the direct transcription-independent apoptotic p53 program at mitochondria. With respect to the relative strength in apoptosis induction in vivo, p53CTB and p53CTM resemble Nucp53 (see Table 1). Mitochondrial p53 uses its DNA-binding domain to inhibit the antiapoptotic BclXL/Bcl2 proteins and to induce BAK and BAX oligomerization and cytochrome c release (1, 42–45). Nuclear magnetic resonance studies confirmed that the BclXL interaction surface on p53 involves the same region that is used to contact DNA (44). In addition, mitochondrial p53 can also directly bind and oligomerize proapoptotic BAK (45). However, in contrast to transcriptionally active p53, mitochondrial p53 does not require tetramerization because its COOH-terminal domain is fully dispensable (9). This potentially provides another important advantage in that mitochondrially targeted p53 might escape dominant negative inhibition by endogenous mutant p53 proteins and ΔNp63/p73 isoforms in tumors. Together with its transcription independence and potential escape from dominant negative interference, our data suggests the possible therapeutic relevance of targeting p53 to mitochondria, perhaps as a synergistic modality with existing p53-based strategies.

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